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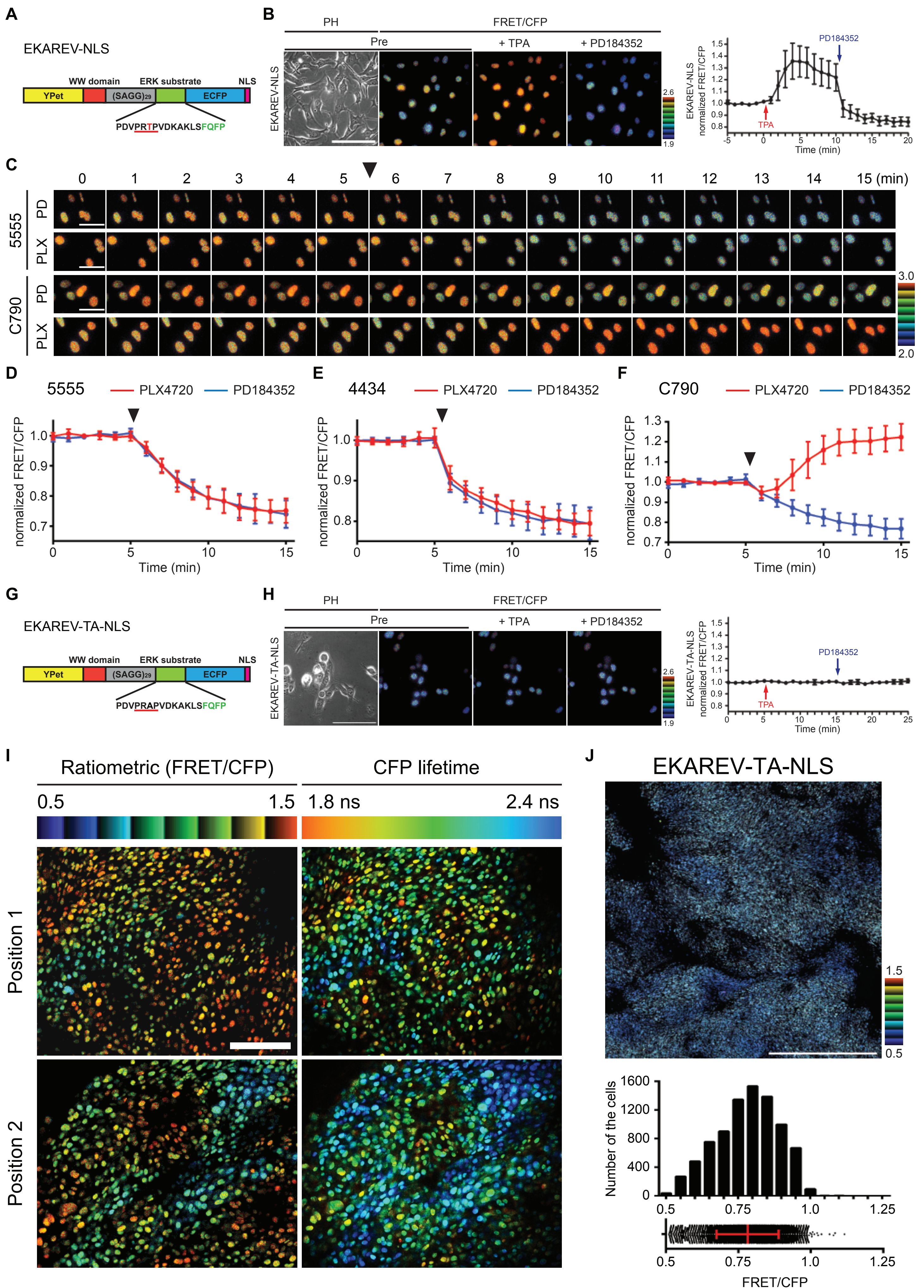
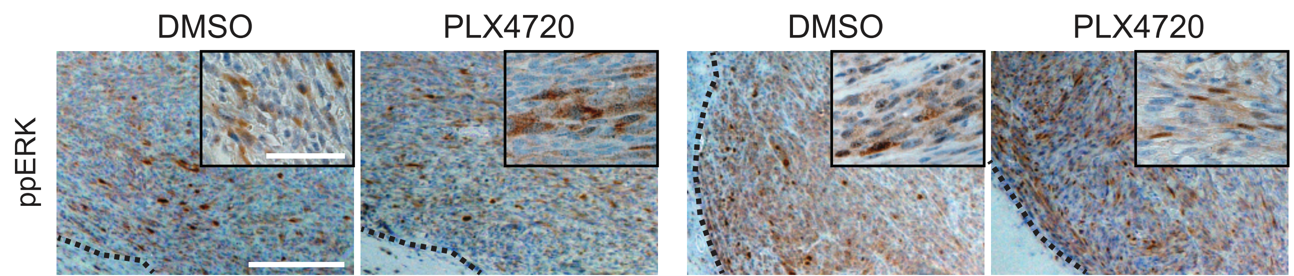
Supplemental Information

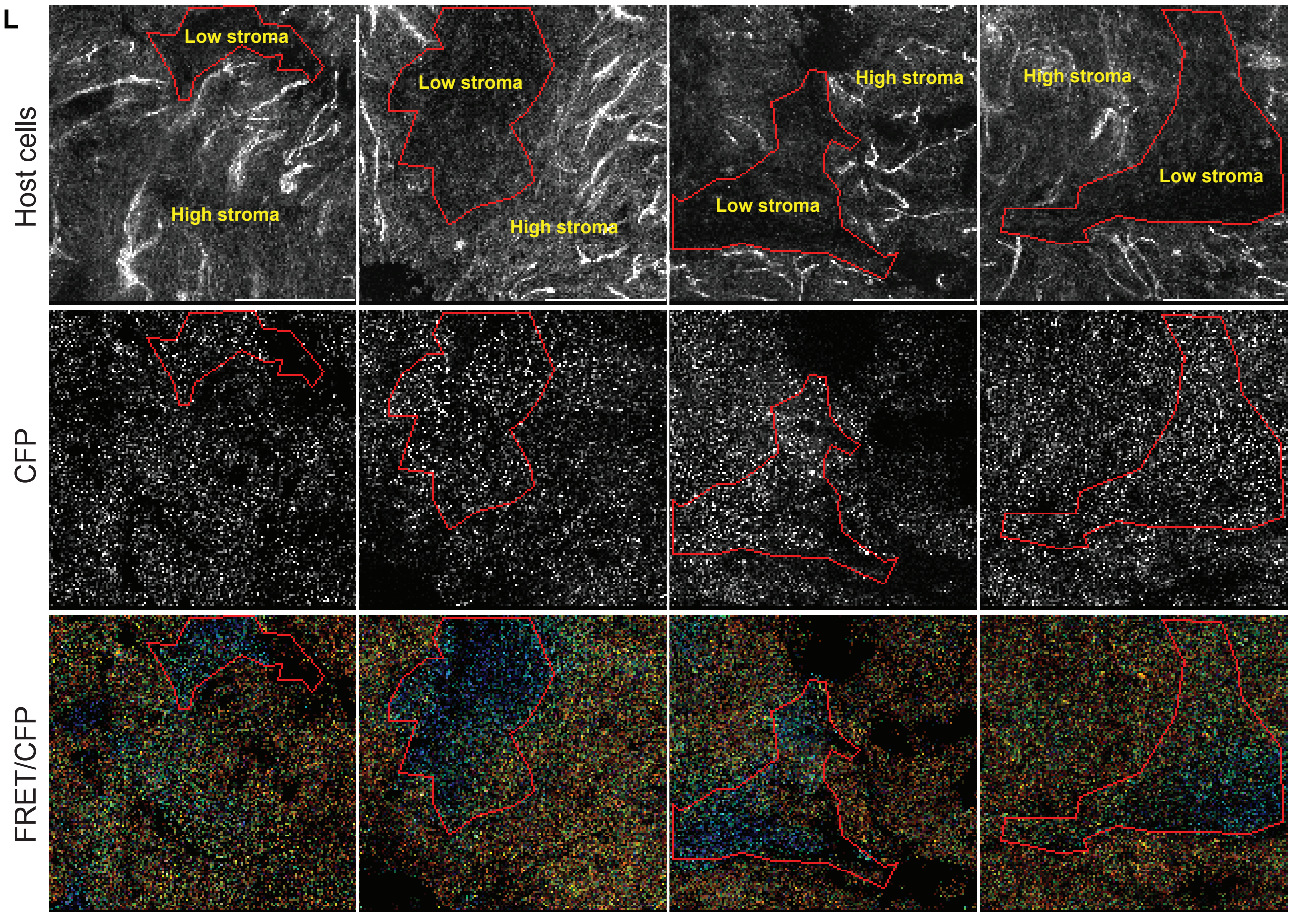
Intravital Imaging Reveals How BRAF Inhibition Generates Drug-Tolerant Microenvironments with High Integrin β1/FAK Signaling

Eishu Hirata, Maria Romina Girotti, Amaya Viros, Steven Hooper, Bradley Spencer-Dene, Michiyuki Matsuda, James Larkin, Richard Marais, and Erik Sahai

Supplemental Data




K
5555
4434
DMSO
PLX4720
DMSO
PLX4720
ppERK


L
Host cells
CFP
FRET/CFP
Low stroma
High stroma
Low stroma
High stroma
High stroma
Low stroma
High stroma
Low stroma

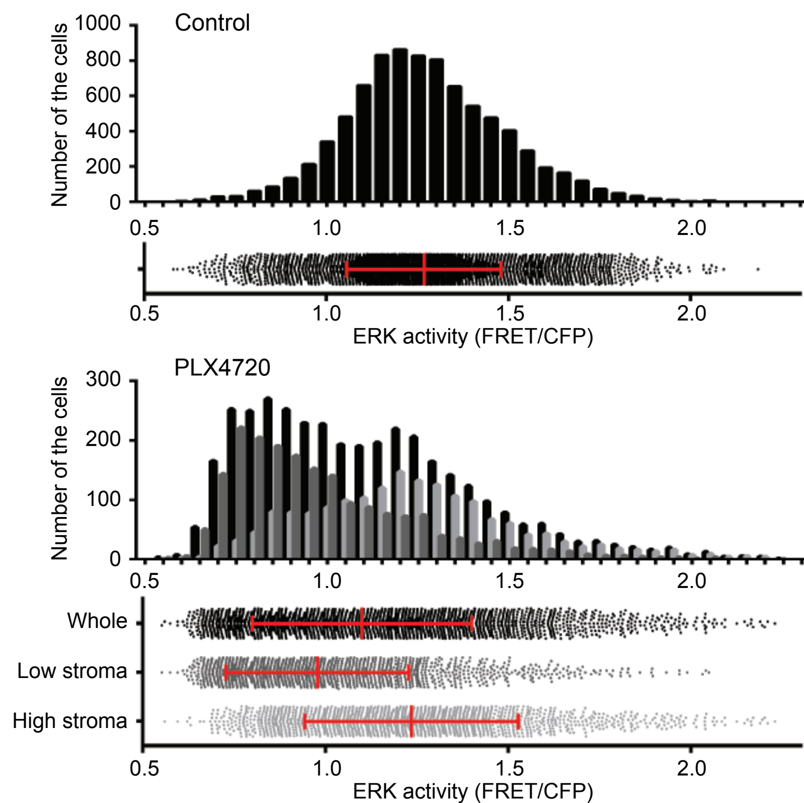
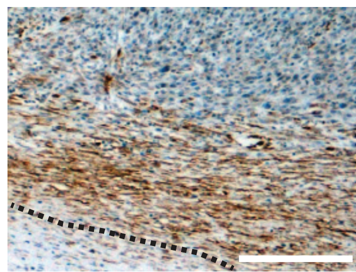
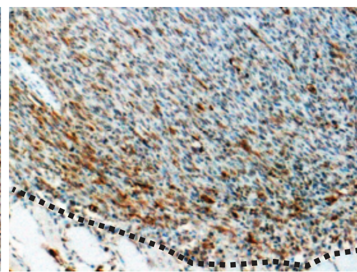
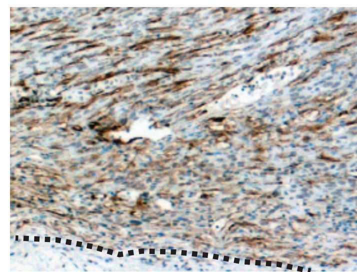
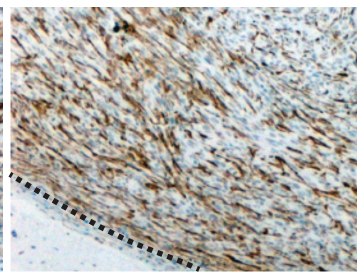

M
4434-EKAREV-NLS
Control
Number of the cells
1000
800
600
400
200
0
0.5
1.0
1.5
2.0
ERK activity (FRET/CFP)
PLX4720
300
200
100
Whole
Low stroma
High stroma

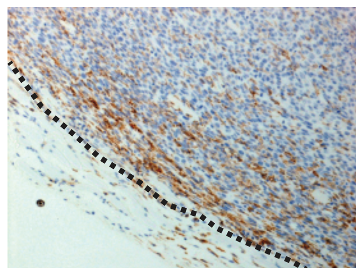
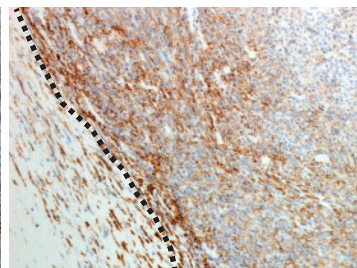
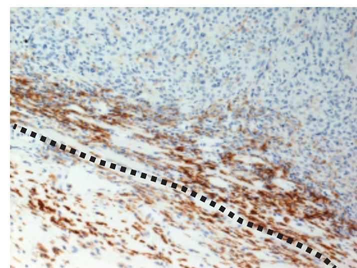
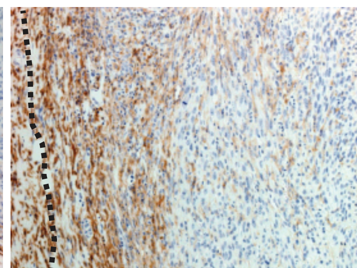
Figure S1. (Related to Figure 1.) Intravital FRET imaging revealed heterogeneous response to a BRAF inhibitor, which is related to stromal cell density.

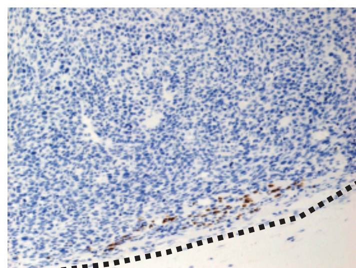
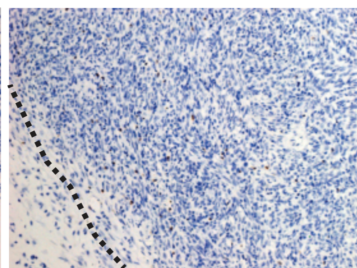
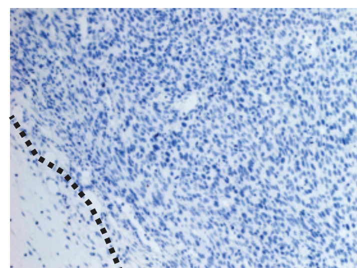
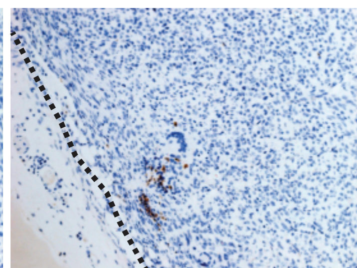
(A) A scheme of EKAREV-NLS biosensor. (B) B16F10 mouse melanoma cells expressing EKAREV-NLS biosensors were treated with 12-O-Tetradcanoyl-phorbol-13-acetate (TPA) (100 nM) and PD184352 (1 μM) at the indicated time points. Images are acquired every 1 min and normalized FRET/CFP ratios were quantified and shown as mean ± SD (n = 10). Scale bar = 100 μm. (C-F) Indicated cells expressing EKAREV-NLS were treated with 1 μM PLX4720 (quantified in red curves) or 1 μM PD184352 (in blue curves) at time = 5 min (indicated by arrowheads). Normalized ERK activities (FRET/CFP) are shown as mean ± SD. Scale bars = 50 μm. (G) A scheme of negative control ERK biosensor (EKAREV-TA-NLS). (H) B16F10 mouse melanoma cells expressing EKAREV-TA-NLS biosensors were treated with TPA (100 nM) and PD184352 (1 μM) at the indicated time points. Images are acquired every 1 min and normalized FRET/CFP ratios were quantified and shown as mean ± SD (n = 10). Scale bar = 100 μm. (I) Same positions of 5555-EKAREV-NLS tumors in C57BL/6 mice were successively imaged and analyzed by intensity-based ratiometric analysis (FRET/CFP) (left panels) and time-correlated single photon counting (TCSPC) FLIM-FRET analysis (right panels). Scale bar = 100 μm. (J) A representative image of 5555-EKAREV-TA-NLS grown subcutaneously in a C57BL/6 mouse, and the distribution (a scatter plot with mean ± SD) and histogram of FRET/CFP are quantified and shown. Scale bar = 500 μm. (K) 5555 and 4434 allografts treated with DMSO (4%) or PLX4720 (25 mg/kg) for 8-14 days were fixed and stained with a phosphor-ERK antibody. Tumor margins are delineated by dashed lines, and enlarged views are also shown as insets. Scale bar = 200 μm and 50 μm (inset). (L) To quantify ERK activity in melanoma cells separately according to the surrounding stromal cell density, we manually set up ROIs depending on the intensity of mTomato (host cells) and defined ‘low stroma’ region (inside the red line in each panel) and ‘high stroma’ region (outside the red line). Scale bar = 500 μm.

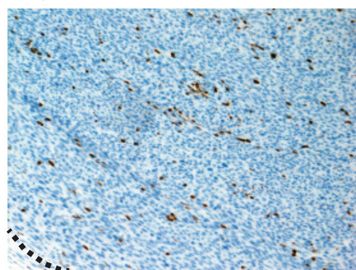
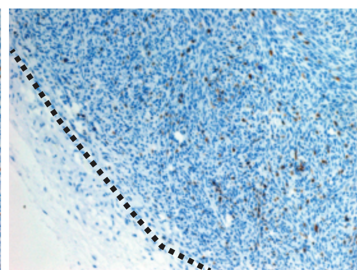
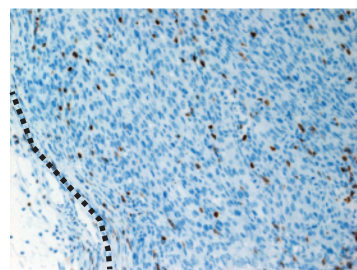
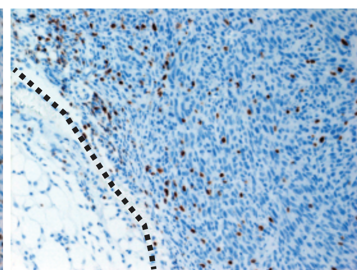
(M) Quantification of ERK activity in skin-flap imaging of 4434-EKAREV-NLS in C57BL/6_ROSA26-mTmG mice, presented in the same manner as in Figure 1F.

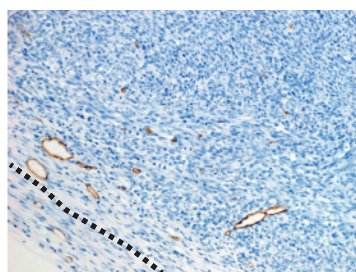
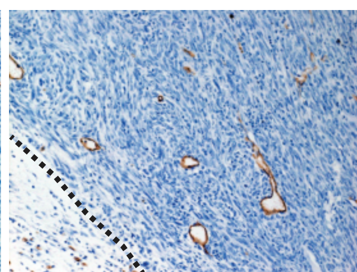
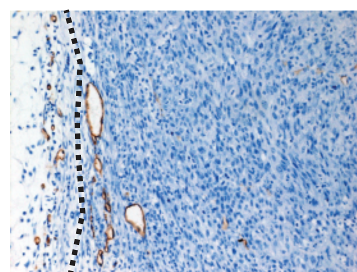
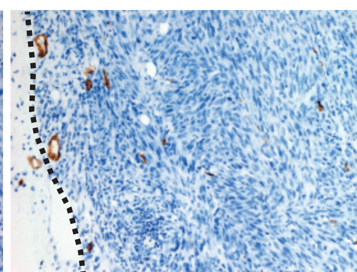
A

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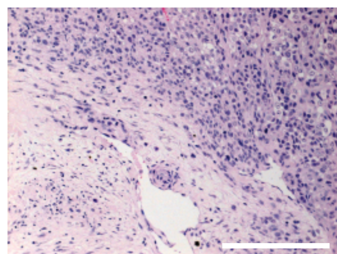
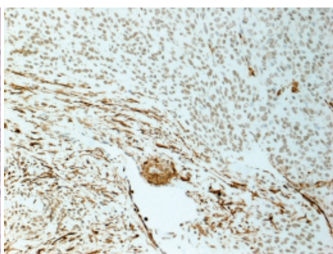
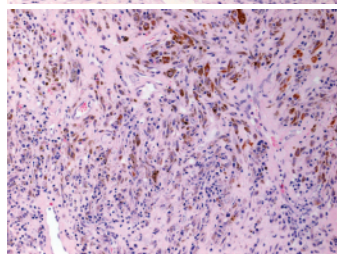
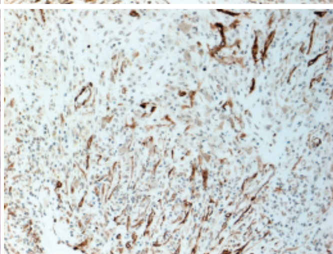

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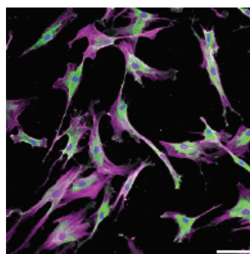
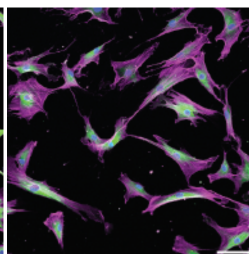
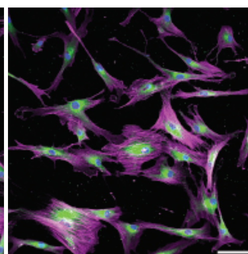
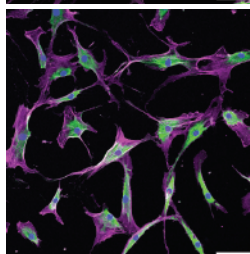
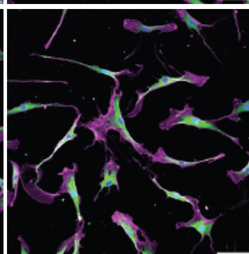
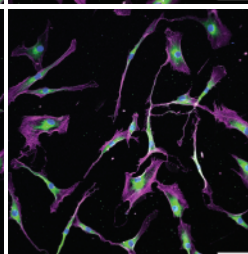







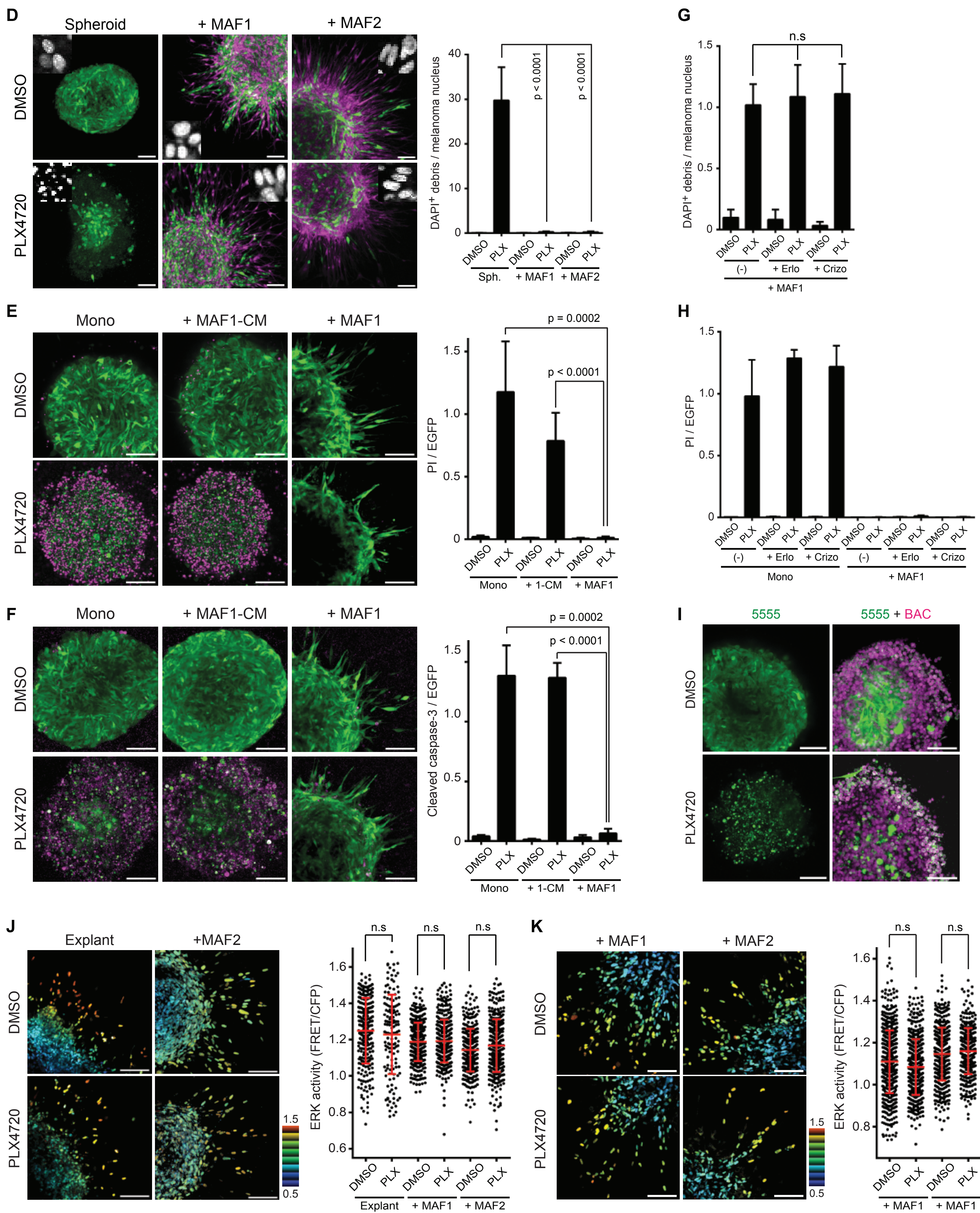

D
Spheroid
+ MAF1
+ MAF2
DMSO
PLX4720
DAPI+ debris / melanoma nucleus
p < 0.0001
p < 0.0001
DMSO PLX DMSO PLX DMSO PLX
Sph. + MAF1 + MAF2
G
n.s
DAPI+ debris / melanoma nucleus
DMSO PLX DMSO PLX DMSO PLX
(-) + Erlo + Crizo
+ MAF1
E
Mono
+ MAF1-CM
+ MAF1
p = 0.0002
p < 0.0001
PI / EGFP
DMSO PLX DMSO PLX DMSO PLX
Mono + 1-CM + MAF1
H
PI / EGFP
DMSO PLX DMSO PLX DMSO PLX DMSO PLX DMSO PLX DMSO PLX
(-) + Erlo + Crizo (-) + Erlo + Crizo
Mono
+ MAF1
F
Mono
+ MAF1-CM
+ MAF1
p = 0.0002
p < 0.0001
Cleaved caspase-3 / EGFP
DMSO PLX DMSO PLX DMSO PLX
Mono + 1-CM + MAF1
I
5555
5555 + BAC
DMSO
PLX4720
J
Explant
+MAF2
DMSO
PLX4720
n.s
n.s
n.s
ERK activity (FRET/CFP)
DMSO PLX DMSO PLX DMSO PLX
Explant + MAF1 + MAF2
K
+ MAF1
+ MAF2
DMSO
PLX4720
n.s
n.s
ERK activity (FRET/CFP)
DMSO PLX DMSO PLX
+ MAF1 + MAF1

Figure S2. (Related to Figure 2.) Melanoma-associated fibroblastg UfY sufficient to induce ERK re-activation and drug tolerance.

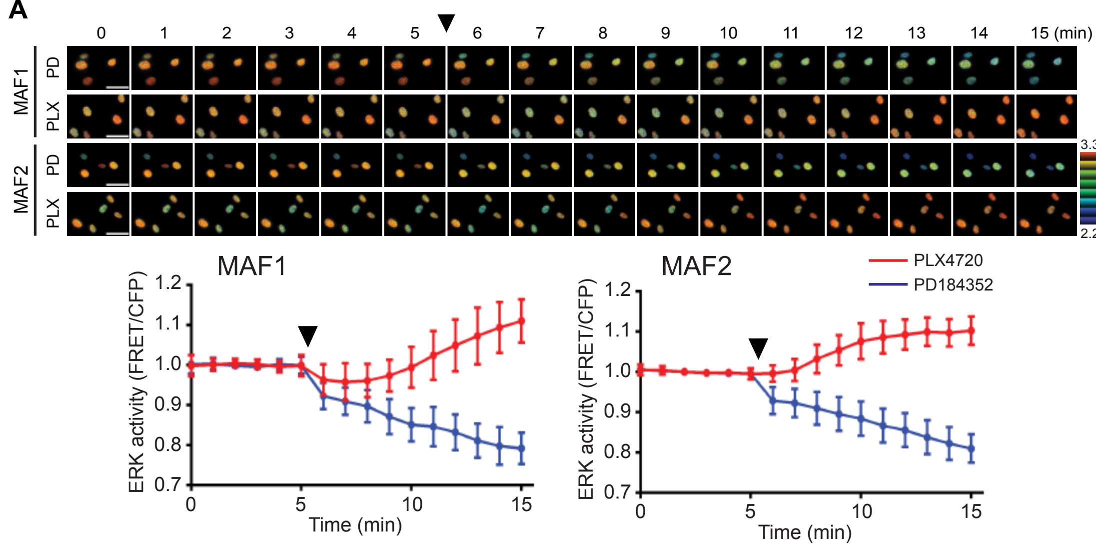
(A) 5555 and 4434 allografts treated with DMSO (4%) or PLX4720 (25 mg/kg) for 8-14 days were fixed and stained with the indicated antibodies. Tumor margins are delineated by dashed lines. Scale bar = 200 μm. (B) Two human melanoma surgical specimens stained with H&E and anti-αSMA antibody. Scale bar = 200 μm. (C) Established MAFs cultured in DMEM are stained with the indicated antibodies. Scale bars = 100 μm. (D) 4434 (green) mono-cultured spheroids or co-cultured spheroids with MAF1/2 (magenta) were cultured in the same manner in Figure 2A, and the cell death was quantified in the same manner in Figure 2B. Scale bars = 100 μm. (E) 5555-mEGFP spheroids cultured with or without MAF1-conditioned media were treated with DMSO (0.1%) or PLX4720 (1 μM) for 24 hr, followed by propidium iodide (PI) staining. PI intensities were quantified and shown as mean ± SD. Scale bar = 100 μm. (F) 5555-mEGFP and MAF1 co-cultured spheroids were treated with DMSO (0.1%) or PLX4720 (1 μM) for 24 hr, fixed and stained with an anti-cleaved caspase-3 antibody. Cleaved caspase-3 signals were quantified and shown as mean ± SD. Scale bars = 100 μm. (G) 5555 and MAF1 co-cultured spheroids were treated with DMSO (0.1%), PLX4720 (1 μM), erlotinib (1 μM), crizotinib (1 μM) or the combination. Cell death was quantified by the ratio of DAPI-positive debris and 5555 nucleus. (mean ± SD). (H) 5555 mono-cultured spheroids or 5555 and MAF1 co-cultured spheroids were treated with DMSO (0.1%), PLX4720 (1 μM), erlotinib (1 μM), crizotinib (1 μM) or the combination. Cell death was quantified by PI staining (mean ± SD). (I) 5555-mEGFP (in green) mono-cultured spheroids or co-cultured spheroids with BAC macrophage cells (labeled with Cherry and shown in magenta) were treated with DMSO (0.1%) or PLX4720 (1 μM) for 24 hr. Scale bars = 100 μm. (J) 5555-EKAREV-NLS tumor explants or co-cultured spheroids with MAF1/2 were embedded into collagen gels and treated with DMSO (0.1%) or PLX4720 (1 μM) for 24 hr. ERK activity in invaded 5555 cells were quantified and shown as scatter plots with mean ± SD. Scale bars = 100 μm. (K) 4434-EKAREV-NLS and MAF1/2-

mCherry co-cultured spheroids were treated in the same manner in (J) and ERK activities were quantified and shown in the same manner in (J). Scale bars = 100 μm.

Movie S1. (Related to Figure 2.) Melanoma-associated fibroblasts provide invasive and pro-survival signals, and the system adapts to the drug within 12 hr.

5555-EKAREV-NLS mono-cultured spheroids (Mono) or co-cultured spheroids with MAF1-mCherry (+ MAF1) were embedded in collagen gels and treated with DMSO (0.1%) or PLX4720 (1 μM). Images were acquired every 5 min for 13 hr. Scale bars = 100 μm.

A



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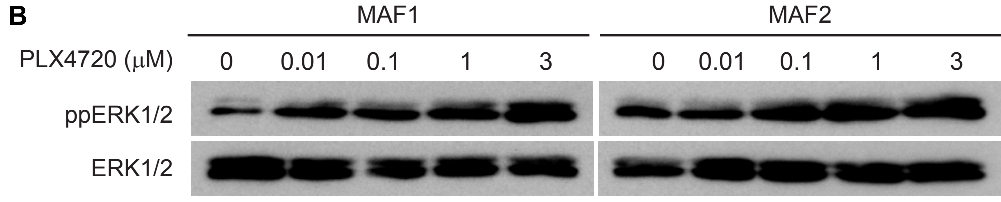

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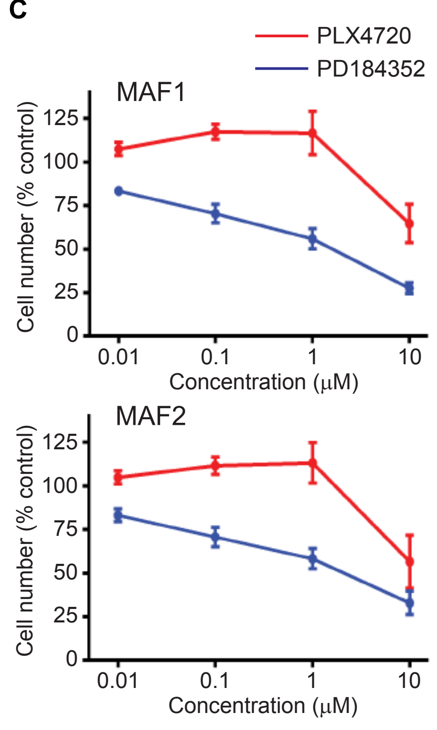

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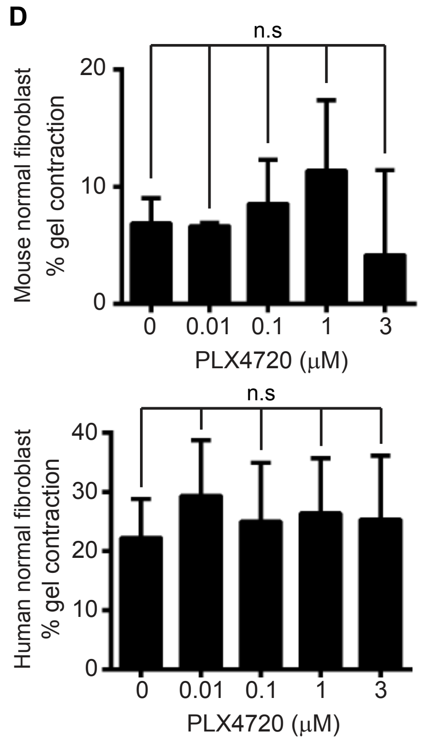

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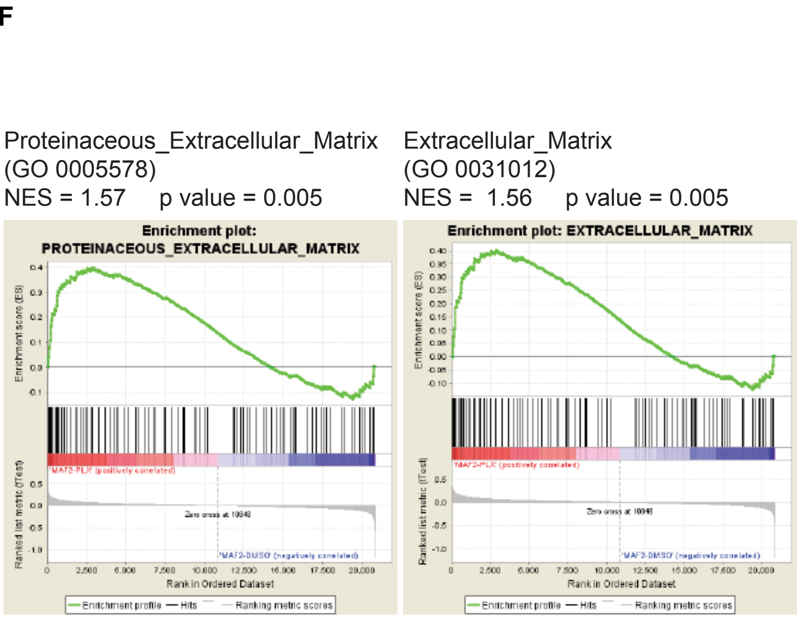
F



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POSTN	1.476500263
COL12A1	1.43913
LAMB1	1.410675
COL4A1	1.353
TNC	1.233553
FN1	1.163538

Soluble factors	
HGF	1.04796
IGF1	1.037288
HBEGF	1.056623221
EGF	0.995187566

Receptor Tyrosine Kinases	
PDGFRA	1.496068778
PDGFRB	1.016803791

G

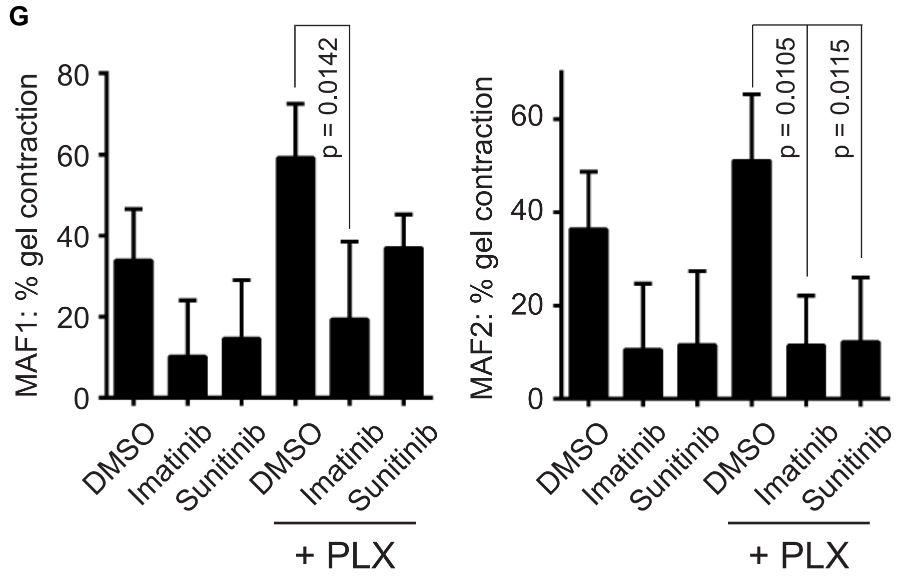


Figure S3. (Related to Figure 3.) PLX4720 paradoxically activates MAFs and induces ECM expression.

(A) Timelapse images of MAF1/2 -EKAREV-NLS, stimulated with PLX4720 (1 μM) or PD184352 (1 μM) at time = 5 min (indicated by arrowheads). ERK activities in MAF1 and MAF2 were quantified and shown as normalized FRET/CFP ratio (n = 10, mean ± SD). Scale bars = 50 μm. (B) Immunoblotting for the indicated proteins in MAF1/2 treated with different concentration of PLX4720 (0–3 μM). (C) Cell viability assay of MAF1 and MAF2 treated with different concentration of PLX4720 (red curves) and PD184352 (blue curves) for 3 days. Data are represented as mean ± SD. (D) Histograms of gel contraction by mouse lung normal fibroblasts and human dermal normal fibroblasts treated with different concentration of PLX4720 (0–3 μM). Data are represented as mean ± SD. (E) 5555-mEGFP and MAF2-mCherry were co-cultured and treated with DMSO (0.1%) or PLX4720 (1 μM) for 24 hr. Cells were trypsinized, sorted according to the expression of EGFP and Cherry, and total RNA was extracted from each component. Gene expression was analyzed with a microarray and the fold change (up-regulated in PLX4720-treated MAF2 compared to DMSO-treated MAF2) of matrix components, soluble factors, and receptor tyrosine kinases of our interest are shown. More details are available as Table S1. (F) Gene set enrichment analysis of PLX4720-treated MAF2 versus DMSO-treated MAF2 with the indicated data sets. (G) Histograms of MAF1 and MAF2 gel contraction with DMSO (0.1%), imatinib (1 μM), sunitinib (1 μM) ± PLX4720 (1 μM) from 3 independent experiments (mean ± SD).

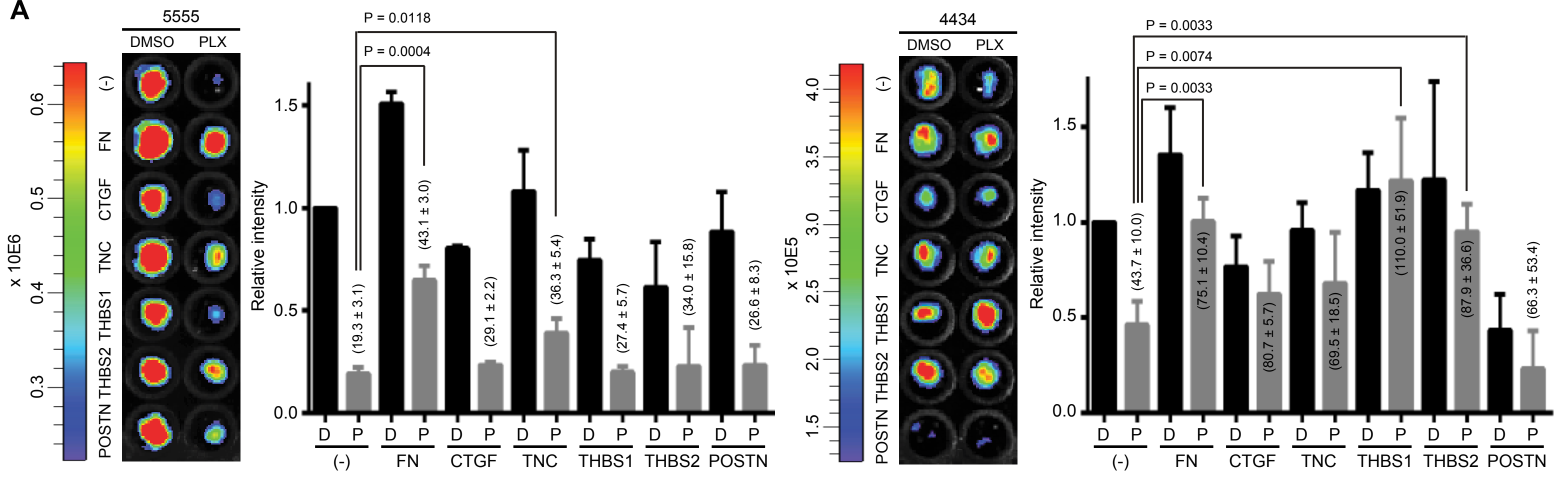
Movie S2. (Related to Figure 3.) PLX4720 paradoxically activate ERK in MAFs.

5555-EKAREV-NLS and MAF1-EKAREV-NLS-mCherry co-cultured spheroids were embedded in collagen gels and treated with PLX4720 (1 μM). Images were acquired every 5 min for 13 hr. White arrows indicate MAF nuclei to be focused on. Scale bars = 100 μm.

Movie S3. (Related to Figure 3.) PLX4720 promotes MAF motility and elongation.

MAF1/2-mCherry embedded in collagen/Matrigel with 10%FBS were treated with DMSO (0.1%) or PLX4720 (1 μM). Images were acquired every 10 min for 13 hr. Scale bars = 100 μm.

Table S1. (Related to Figure 3. Provided as an Excel file.) Summary of the microarray.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5555
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(-) FN CTGF TNC THBS1 THBS2 POSTN
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P = 0.0004
Relative intensity
(19.3 ± 3.1)
(43.1 ± 3.0)
(29.1 ± 2.2)
(36.3 ± 5.4)
(27.4 ± 5.7)
(34.0 ± 15.8)
(26.6 ± 8.3)
4434
x 10E5
P = 0.0033
P = 0.0074
(43.7 ± 10.0)
(75.1 ± 10.4)
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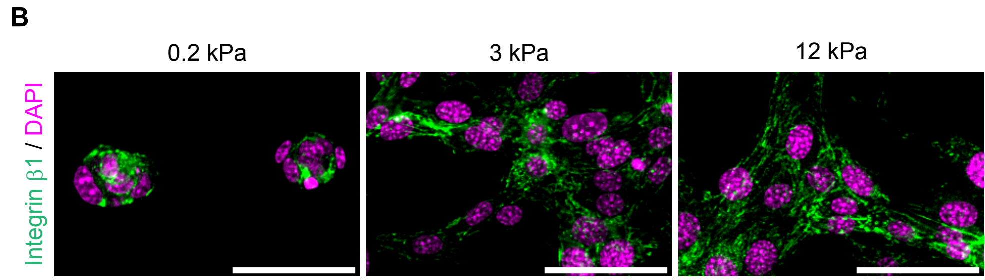
(110.0 ± 51.9)
(87.9 ± 36.6)
(66.3 ± 53.4)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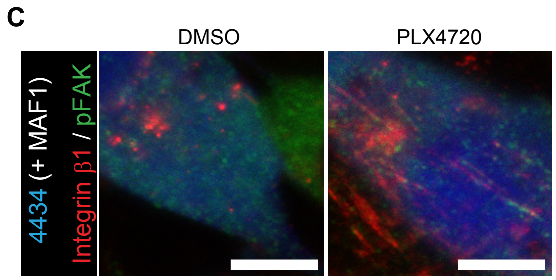
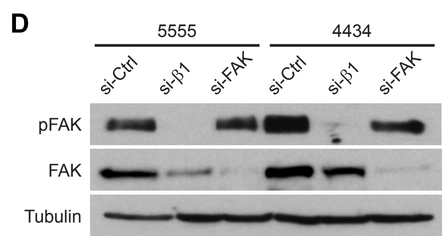
12 kPa
Integrin β1 / DAPI


C
DMSO
PLX4720
4434 (+ MAF1)
Integrin β1 / pFAK


D
5555
4434
si-Ctrl
si-β1
si-FAK
pFAK
FAK
Tubulin

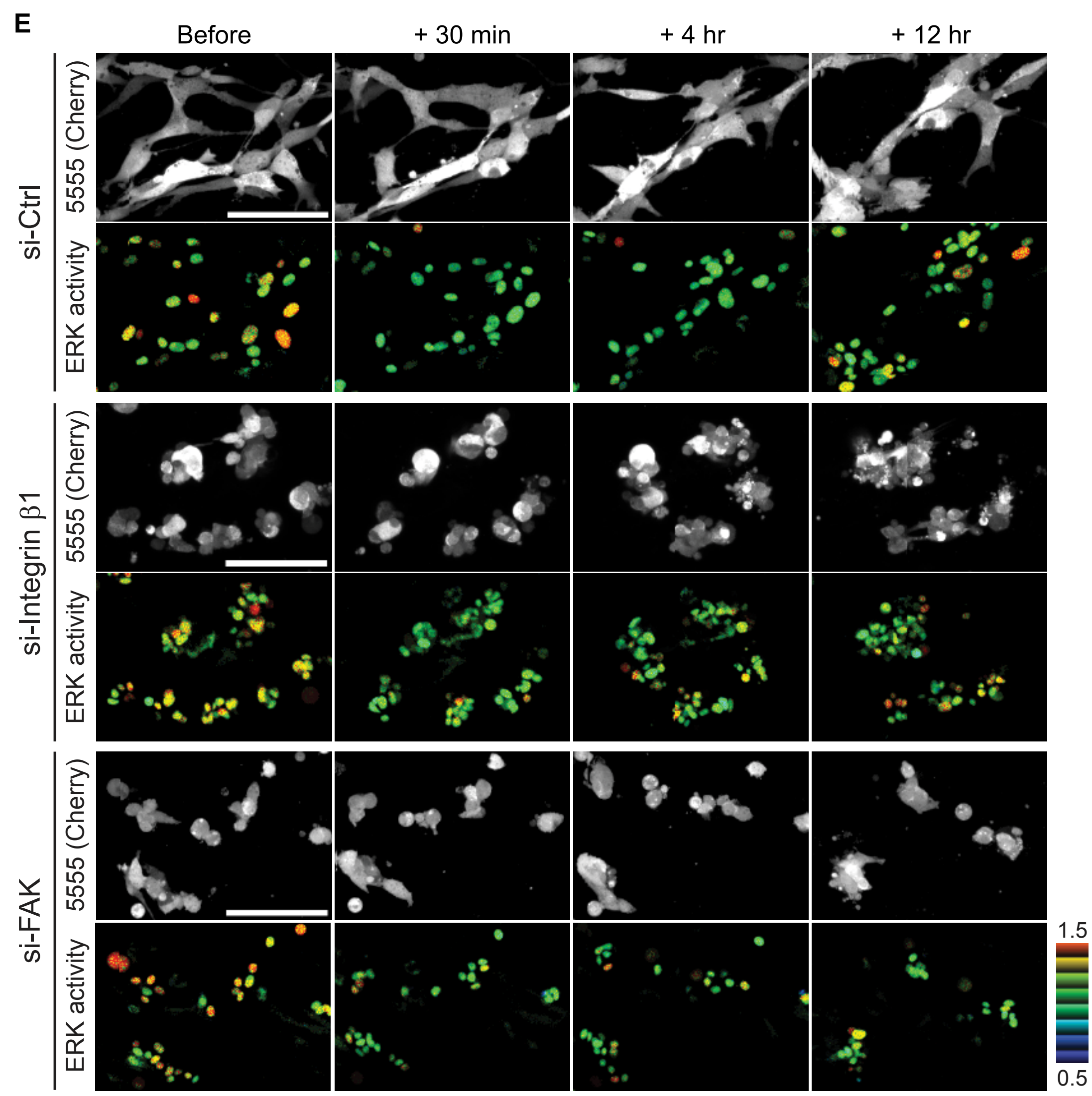
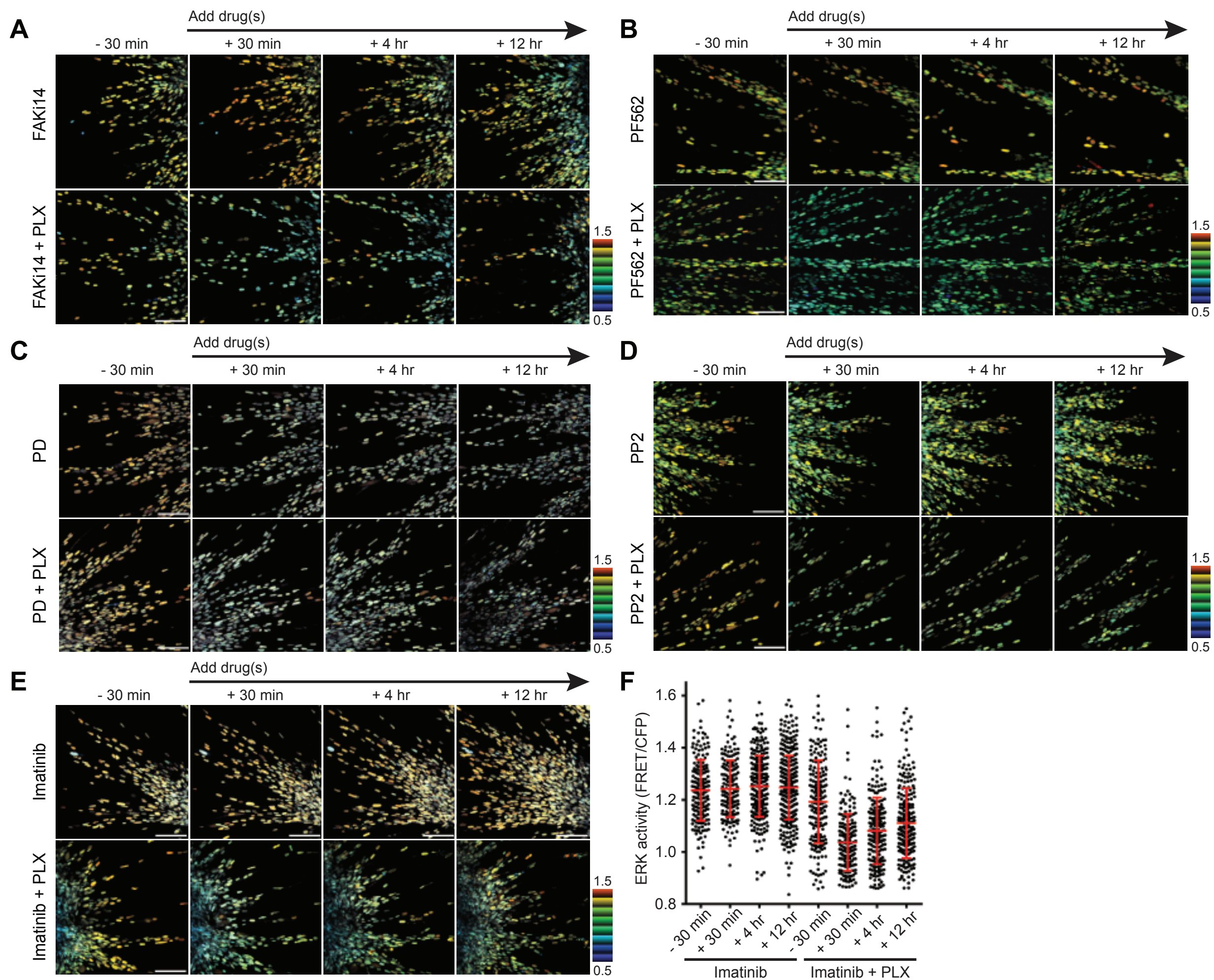

E
Before
+ 30 min
+ 4 hr
+ 12 hr
si-Ctrl
si-Integrin β1
si-FAK
5555 (Cherry)
ERK activity
1.5
0.5

Figure S4. (Related to Figure 4.) ECM provides drug tolerant microenvironment through integrin β1-FAK signaling.

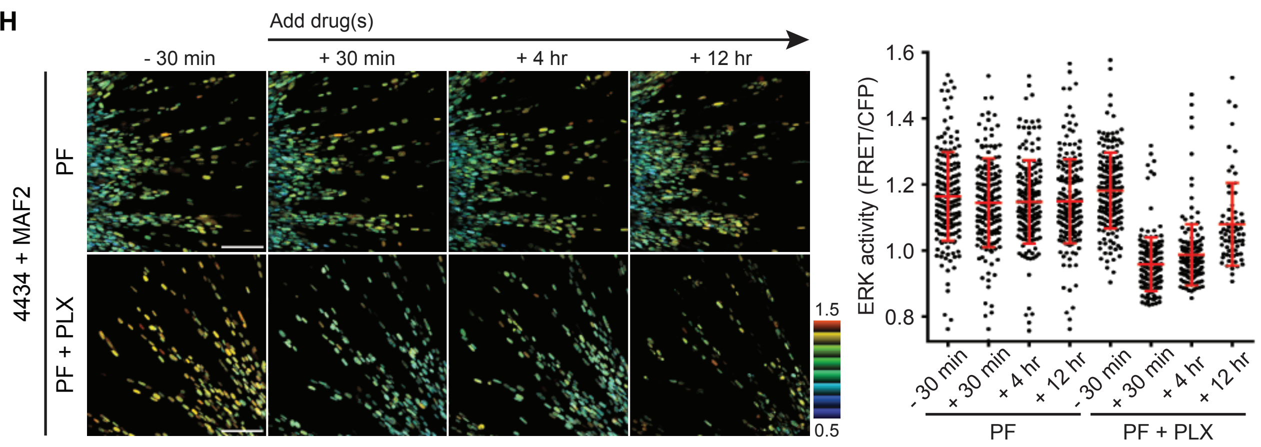
(A) 5555 and 4434 cells expressing firefly luciferase were seeded onto 96-well plate coated with collagen I (100 mg/ml) ± fibronectin (20 mg/ml), CTGF (20 mg/ml), tenascin-C (20 mg/ml), thrombospondin-1 (20 mg/ml), thrombospondin-2 (20 mg/ml), or periostin (20 mg/ml), and treated with DMSO (0.1%) or PLX4720 (1 μM) for 24 hr, followed by bioluminescence imaging. Signals were quantified and shown in the graphs (n = 3, mean ± SD). (B) 5555 cells cultured on the fibronectin-coated gels with the indicated stiffness were fixed and stained with an anti-active integrin β1 antibody (9EG7) (in green) and DAPI (in magenta). Scale bars = 50 μm. (C) 4434 cells (blue) were co-cultured with MAF1 (not labeled) and treated with DMSO (0.1%) or PLX4720 (1 μM) for 24 hr. After fixation, cells were double-stained with an anti-active integrin β1 antibody (9EG7) (red) and an anti-phospho-FAK antibody (green). Scale bars = 20 μm. (D) Immunoblotting for the indicated proteins in 5555 and 4434 cells transfected with the indicated siRNAs. (E) 5555-EKAREV-NLS-mCherry transfected with the indicated siRNAs were seeded onto collagen gels with MAF1 (not labeled) and imaged before and after PLX4720 treatment. Scale bars = 100 μm.



G

	DMSO		PLX4720	
(-)	p = 0.1022	Before: 1.180 ± 0.010, n=238 12 hr: 1.203 ± 0.010, n=279 ND: 1.019 ± 0.008	p = 0.1006	Before: 1.176 ± 0.010, n=189 12 hr: 1.152 ± 0.011, n=200 ND: 0.982 ± 0.009
Imatinib	p = 0.3255	Before: 1.236 ± 0.009, n=187 12 hr: 1.247 ± 0.006, n=385 ND: 1.008 ± 0.005	p < 0.0001	Before: 1.192 ± 0.014, n=196 12 hr: 1.111 ± 0.009, n=248 ND: 0.931 ± 0.007
PF573228	p = 0.0099	Before: 1.205 ± 0.009, n=160 12 hr: 1.172 ± 0.009, n=130 ND: 0.973 ± 0.008	p < 0.0001	Before: 1.167 ± 0.007, n=199 12 hr: 1.057 ± 0.018, n=62 ND: 0.906 ± 0.015
PF562271	p = 0.0270	Before: 1.237 ± 0.009, n=138 12 hr: 1.266 ± 0.009, n=114 ND: 1.023 ± 0.007	p < 0.0001	Before: 1.176 ± 0.007, n=172 12 hr: 1.120 ± 0.008, n=123 ND: 0.949 ± 0.006
FAK inhibitor 14	p = 0.4734	Before: 1.181 ± 0.007, n=185 12 hr: 1.188 ± 0.008, n=194 ND: 1.006 ± 0.006	p < 0.0001	Before: 1.143 ± 0.009, n=168 12 hr: 1.033 ± 0.016, n=75 ND: 0.903 ± 0.014
Dasatinib	p < 0.0001	Before: 1.186 ± 0.005, n=243 12 hr: 1.136 ± 0.005, n=288 ND: 0.958 ± 0.004	p < 0.0001	Before: 1.221 ± 0.008, n=188 12 hr: 1.000 ± 0.006, n=277 ND: 0.819 ± 0.005
PP2	p = 0.1988	Before: 1.160 ± 0.011, n=115 12 hr: 1.179 ± 0.010, n=179 ND: 1.016 ± 0.009	p < 0.0001	Before: 1.186 ± 0.009, n=129 12 hr: 0.990 ± 0.006, n=134 ND: 0.835 ± 0.005
PD184352	p < 0.0001	Before: 1.233 ± 0.007 n=237 12 hr: 0.965 ± 0.004, n=260 ND: 0.783 ± 0.004	p < 0.0001	Before: 1.218 ± 0.008, n=236 12 hr: 0.998 ± 0.006, n=233 ND: 0.801 ± 0.004

H



I

	DMSO	PLX4720
(-)	NA	p = 0.2363 Before: 1.075 ± 0.123, n=225 12 hr: 1.061 ± 0.117, n=201 ND: 0.987 ± 0.108
PF573228	p = 0.2698 Before: 1.164 ± 0.134, n=185 12 hr: 1.149 ± 0.127, n=206 ND: 0.987 ± 0.109	p < 0.0001 Before: 1.182 ± 0.115, n=182 12 hr: 1.079 ± 0.126, n=70 ND: 0.913 ± 0.106

J

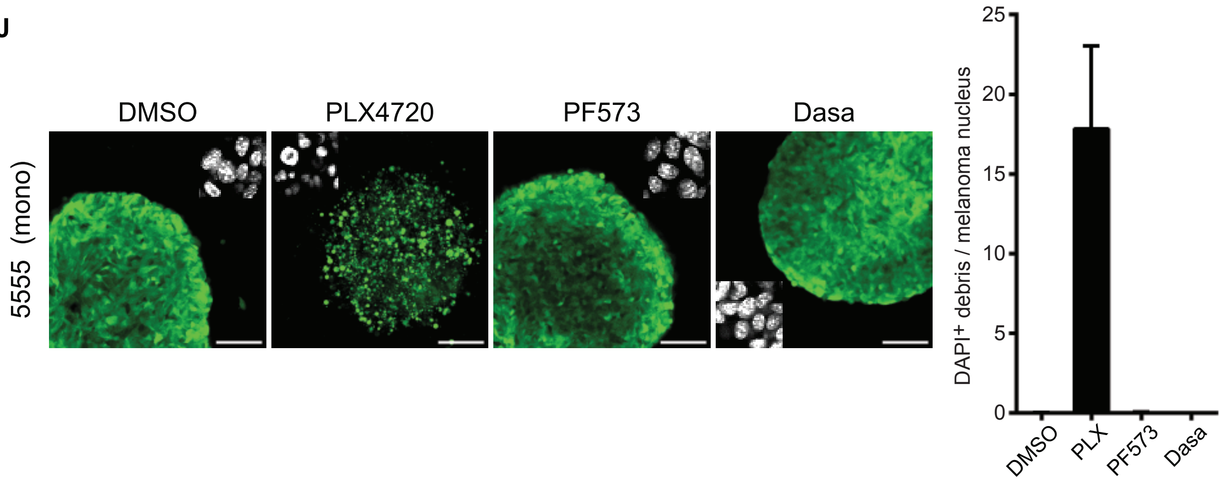


Figure S5. (Related to Figure 5.) Simultaneous inhibition of BRAF and FAK/Src effectively suppresses ERK reactivation in melanoma cells.

(A-F) Images of 5555-EKAREV-NLS and MAF1-mCherry co-cultured spheroids treated with FAKi14 (1 μM), PF562271 (1 μM), PP2 (1 μM), PD184352 (1 μM), imatinib (1 μM) ± PLX4720 (1 μM). ERK activities in imatinib ± PLX4720 treated cells were quantified at the indicated time points and shown in (F) as scatter plots with mean ± SD. Scale bars = 100 μm. (G) Statistical analysis of ERK activity in 5555-EKAREV-NLS and MAF1-mCherry co-cultured spheroids treated with the indicated drug combinations. The values indicate mean ± SD. ND; normalized ERK/MAPK activity 12 hr after addition of indicated drugs, normalized to 30 min before treatment. Red color gradient indicates the effect of the drugs on ERK activity after 12 hr. (H) Images of 4434-EKAREV-NLS and MAF2-mCherry co-cultured spheroids treated with PF573228 (1 μM) ± PLX4720 (1 μM). ERK activities were quantified at the indicated time points and shown as scatter plots with mean ± SD. Scale bars = 100 μm. (I) Statistical analysis of ERK activity in 4434-EKAREV-NLS and MAF2-mCherry co-cultured spheroids, described in the same manner in (G). (J) 5555-mEGFP mono-cultured spheroids were embedded into collagen gels and treated with 0.1% DMSO, PLX4720 (1 μM), PF573228 (1 μM), or dasatinib (200 nM) for 48 hr. Representative DAPI-staining images are also shown in each panel, and the ratio of DAPI-positive debris and melanoma nucleus was calculated and shown as mean ± SD. Scale bars = 100 μm.

Movie S4. (Related to Figure 5.) Simultaneous inhibition of BRAF and FAK/Src abolishes ERK reactivation and effectively induced cell death in 5555 cells.

5555-EKAREV-NLS and MAF1-mCherry co-cultured spheroids were embedded in collagen gels and treated with PF573228 (1 μM) ± PLX4720 (1 μM) or dasatinib (200 nM) ± PLX4720 (1 μM). Images were acquired every 5 min for 13 hr. Scale bars = 100 μm.

A

A375 | WM266.4

DMSO | PLX4720 | DMSO | PLX4720

ppERK

ERK

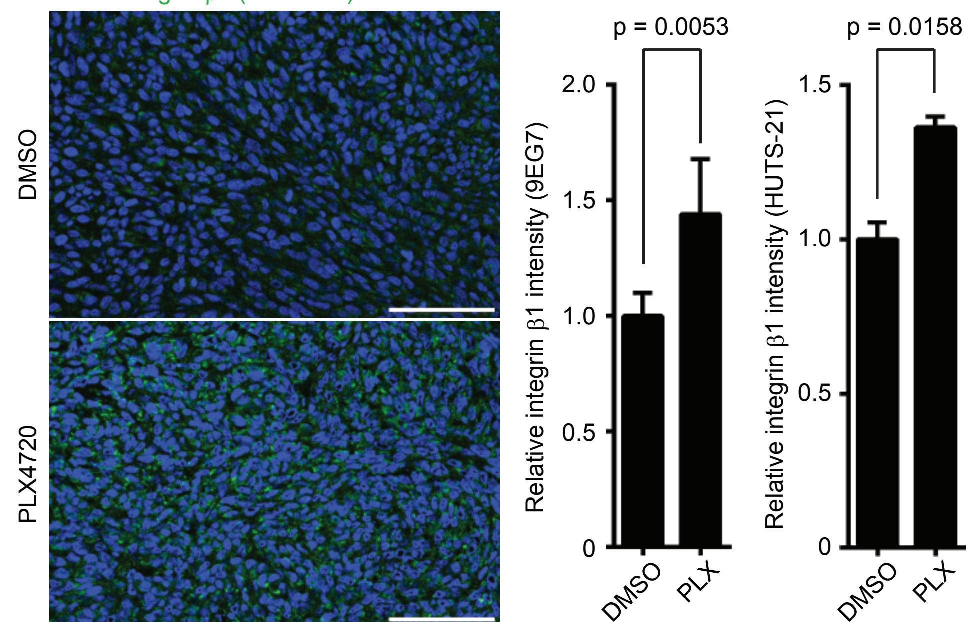
pSFK

H&E

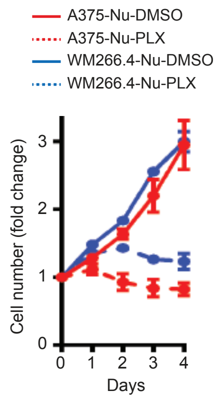
Gomori

Fibronectin

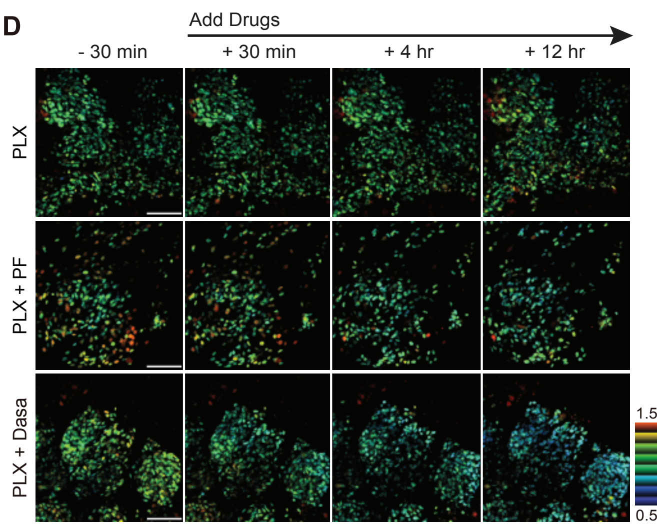
B

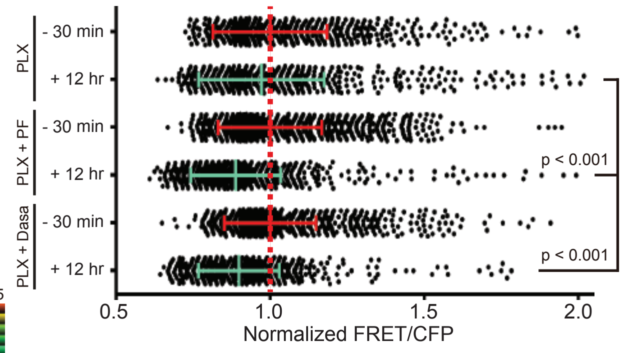


C



D





E

Endomucin αSMA F4/80 S100A9 (2B10)

DMSO

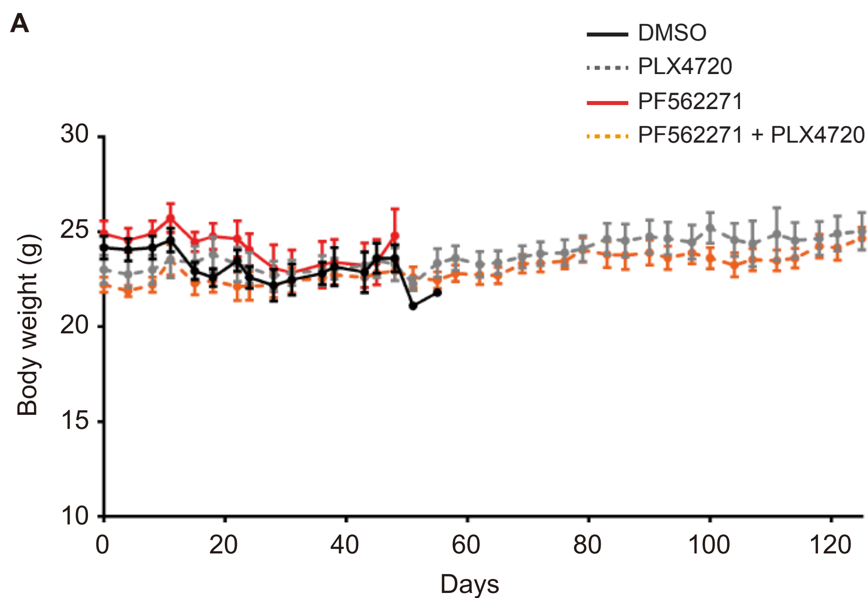
PLX4720

PF562271

PF562271 + PLX4720

Figure S6. (Related to Figure 6.) GhUV`Y`X]gYUgY`cb`6F5:`]b\]V]hcf`hfYUhaYbh]g UggcW]UhYX`k]h\`UhYfYX`97A`UbX`giddcfhYX`Vm:5?`g][bU]b["

(A) A375 and WM266.4 xenograft tumors grown subcutaneously in nude mice treated with DMSO (4%) or PLX4720 (25 mg/kg) were fixed and stained with the indicated methods/antibodies. Arrows in Gomori trichrome staining indicate thick collagen fibers (bright blue) in PLX4720 treated tumors. Scale bars = 200 μm and 20 μm (insets). (B) Frozen sections of A375 tumors treated with DMSO (4%) or PLX4720 (25 mg/kg) for 15 days were stained with two different anti-integrin β1 antibodies (9EG7 and HUTS-21). Integrin β1 intensities were quantified and shown as mean ± SD. Scale bars = 100 μm. (C) In vitro growth curves of A375 and WM266.4 cells re-established from mouse xenograft treated with PLX4720 (25 mg/kg) for 15 days. Data are represented as mean ± SD. (D) Representative FRET images of A375 explants treated with the indicated drugs in 3D collagen gels. The explants were established from mice treated with PLX4720 for 10-13 days. ERK activities at time -30 min and +12 hr were quantified and normalized to before treatment (-30 min) in each treatment group. At least 6 explants from 2 mice were analyzed in each group and the data are shown as scatter plots with mean ± SD. Scale bars = 100 μm. (E) Immunohistochemical staining with the indicated antibodies of A375 tumors treated with DMSO (4%), PLX4720 (25 mg/kg), PF562271 (50 mg/kg) or the combination for 25 - 29 days. Scale bars = 200 μm.


A
DMSO
PLX4720
PF562271
PF562271 + PLX4720
Body weight (g)
30
25
20
15
10
0
20
40
60
80
100
120
Days

B
Patient #1 (PD, Treat = 1 month)
Patient #4 (PR, PFS = 3 months, Treat = 16 months)
Before
After prog.
Before
After prog.
H&E
Gomori
αSMA
ppERK
pSrc

Figure S7. (Related to Figure 7.) Alterations in tumor stroma and ECM in vemurafenib-treated patients.

(A) Body weight curves of PDX-carrying mouse treated with DMSO (4%), PLX4720 (45 mg/kg), PF562271 (50 mg/kg) or the combination. Data are represented as mean ± SEM. (B) H&E, Gomori trichrome, anti-αSMA, anti-phospho-ERK and anti-pSrc staining in paired surgical specimens (before vemurafenib treatment and after progression on drug treatment). Scale bars = 100 μm. PD; progressive disease, PR; partial response, PFS; progression free survival. See also Table S2 for further clinical information. Note: the after progression sample of patient #4 has high levels of melanin (very dark brown), this should not be confused with the lighter brown immuno-staining for alphaSMA, phospho-ERK and phospho-Src.

Table S2. (Related to Figure 7. Provided as an Excel file.) Clinical information of melanoma specimens for a PDX mouse model and histopathological analysis.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Isolation and immortalization of human melanoma associated fibroblasts

Melanoma-Associated Fibroblasts (MAFs) were obtained under approval of the UK Research Ethics Committee 08/H0801/129. MAF1 were cultured from a melanoma metastasis behind knee (popliteal fossa met.) removed from a patient at the Royal Marsden Hospital, London. MAF2 were cultured from a melanoma metastasis (BRAF wt) removed from a patient with progressive disease following temozolomide and ipilumimab treatment at the Royal Marsden Hospital, London. Both MAF lines were established with fibroblast purification procedures, as reported previously (Calvo et al., 2013). Briefly, a sample was cut into small pieces and collagenase/dispase digested. After filtering the undigested tissue, the solution was serial centrifuged and the final pellets were re-suspended in DMEM (Invitrogen) with 10%FBS, 1% ITS (Invitrogen) and seeded on a culture dish. After 30 min, fibroblasts have already adhered to the dish while other cellular types remain in suspension. When purified fibroblasts were obtained after repetition of these procedures, cells were immortalized by hTERT introduction with retroviral vectors (Gaggioli et al., 2007).

Longitudinal intravital tumor imaging through an imaging window

All animal experiments were kept in accordance with UK regulations under project licence PPL/80/2368. Surgical procedures for setting up an imaging window was reported previously. 10 Melanoma spheroids (each containing 20,000 cells) were suspended in Matrigel (BD Biosciences) and injected beneath the window set up on the right lower back of C57BL/6 mice. After 5–10 days, the animals were anesthetized and tumor spheroids were imaged longitudinally at the indicated time points with Zeiss 780 inverted microscope.

Tumor explant and spheroid preparation

Tumor explants were prepared by cutting out subcutaneous tumors or experimentally-induced lung metastasis tumors, chopping into pieces (< 1 mm). Melanoma/MAF spheroids (20,000 cells for melanoma spheroids and 10,000 cells each for melanoma/MAF mixed spheroids) were prepared by a droplet culture method as reported elsewhere. Explants/spheroids were embedded in collagen type I gel (BD Biosciences) at a concentration of approximately 4.2 mg/ml and cultured for subsequent experiments.

FRET microscopy and image processing

For epifluorescence FRET microscopy, cells were plated on 35-mm glass-base dishes, maintained in phenol red–free DMEM with 1% FBS and imaged through a 60x oil-immersion objective lens on an Nikon TE2000 microscope (Nikon) equipped with a cooled CCD camera, CoolSNAP HQ (Roper Scientific), and controlled by MetaMorph software (Universal Imaging, West Chester, PA). For the dual-emission ratio imaging of the FRET biosensors, we used a 440AF21 excitation filter, a 455DRLP dichroic mirror, and two emission filters, 480AF30 for CFP and 535AF26 for FRET (Omega Optical, Newton, MA). After background subtraction, the ratio image of FRET to CFP was created with MetaMorph software, and the results were used to represent the FRET efficiency. All ratiometric FRET images are shown in intensity-modulated display (IMD) mode with 32-intensity in 8-ratio, and the gradient of the color bar shows the activity of ERK/MAPK, with higher activity depicted in red and lower activity in blue. For two-photon excitation microscopy (TPEM), animals or samples were kept on a heated stage or in an incubation chamber and imaged through a 20× dry objective lens (Nikon) with an Zeiss 780 inverted microscope equipped with a Laser Diode, an Argon Laser, an HeNe Laser and a Chameleon Ti:sapphire Laser (Coherent Inc.). For two-photon excited FRET imaging, we used the excitation wavelength of 820 nm through an IR-cut filter, MBC760+, and the emission light was separated by beam splitters into 463-506 nm for CFP and 515-559 nm for FRET. Images were acquired as 12-bit images and analyzed with

MetaMorph software as described above, and FRET/CFP in each cell (nucleus) was calculated in a single section using an intensity threshold of 200 A.D.U. for ERK activity quantification. For timelapse FRET imaging of cultured spheroids, images were acquired every 5 min for up to 13 hr (157 serial images), and analyzed in the same manner, but for quantification of FRET/CFP we used a stacked image of 5 sections (4 μm distance each) so as not to miss the information from motile cells. For FRET image presentation, the original images were analyzed with Imaris Software (Bitplane AG, Zürich, Switzerland) and reconstructed into three-dimensional images, or simply presented as maximum-stack images on Metamorph software. The collagen signal was imaged using second harmonic generation in response to an 820 nm pulsed Ti:Sapphire laser. For other fluorescent dye/protein imaging, DAPI was excited with 405 nm, EGFP with 488 nm, and Tomato and Cherry with 561 nm wavelength, using standard beam splitter setting.

ECM-remodeling assay

To assess force-mediated matrix remodeling (Calvo et al., 2013), 25,000 (MAF1) or 50,000 (MAF2) cells were embedded in 100 µL of Collagen I / Matrigel and seeded on a 24-well glass bottom plate. Once the gel was set, cells were maintained DMEM with 10%FBS, 1% PenStrep and 1% ITS. Gel contraction values were measured after 2 days, and the percentage of contraction was calculated using the formula 100 × (well diameter – gel diameter) / well diameter.

TCSPC FLIM-FRET

FLIM experiments were performed successively with the same two-photon microscope used for CFP/FRET imaging. The Ti:sapphire mode-locked laser (80 MHz) was tuned to 820 nm for CFP excitation and CFP fluorescence emissions were collected through a beam splitter, FT455, and a band pass filter, BP480/40 (Zeiss), using a photon counting photomultiplier tube (HPM-100 hybrid PMT, Becker and Hickl GmbH) and a time-correlated single photon counting (TCSPC) module (SPC-

830, Becker and Hickl GmbH). CFP lifetime was analyzed fitting to a triple-exponential decay curve (Becker et at., 2003 and The bh TCSPC Handbook Fourth Edition), and amplitude-weighted mean lifetime was calculated to create a color-scaled lifetime image, ranging from 1.8 ns (red) – 2.4 ns (blue).

Cell proliferation/viability assay

2 x 10^5 cells in DMEM with 1% FBS were seeded onto 6-well plate and the numbers of the cells were counted by using ViCell Automated Cell Counter (Beckman Coulter) with or without the drugs of interest.

Calculation of DAPI-positive debris/nucleus in spheroid invasion assay

Images were analyzed on Metamorph software. ROIs were set up at the border of spheroids/explants where both the signals from DAPI and GFP (melanoma cells) are detectable. The number of melanoma nucleus was counted manually inside the ROIs, followed by semi-automated counting of DAPI-positive debris: DAPI-staining images are background-subtracted and processed to binary coded > 1000 ADU. 2 ROIs were set in a single spheroid/explant covering the border region, and at least 6 ROIs from 3 different spheroids/explants were analyzed.

Fluorescence-activated cell sorting and microarray analysis.

5555-mEGFP and MAF2-mCherry were co-cultured on 15 cm culture dish with DMSO (0.1%) or LX4720 (1 μM) for 24 hr. Then, cells were trypsinized, re-suspended into PBS with or without PLX4720 (1 μM), and sorted according to the expression of EGFP and Cherry by FACSAria (Becton Dickinson). Cells were directly collected into lysis buffer (RNeasy mini kit, Qiagen) and total RNA was extracted from each fraction. RNA was then processed in collaboration with the Bart's and London Centre for Genome Research (Illumina bead array platform – details available on request). For gene-set enrichment analyses (GSEA), array data was processed and analysed using the Gene-set enrichment analysis software

developed by the Broad Institute of MIT and Harvard (USA) and available at www.broadinstitute.org, following the program guidelines.

ECM screening

96-well glass bottom plates were coated with Collagen I (100mg/ml) +/- Fibronectin (20 mg/ml), CTGF (20 mg/ml), TNC (20 mg/ml), TSP-1 (20 mg/ml), TSP-2 (20 mg/ml), or POSTN (20 mg/ml). 5555/4434 cells stably expressing firefly luciferase were seeded on the plates, cultured overnight. 24 hr after treatment with DMSO or PLX4720, D-Luciferin (150 μg/ml) was added into the wells and the plates were imaged under IVIS Spectrum.

Cell viability assay on PAA/Bis-AA gels with defined stiffness.

PAA/Bis-AA gels with defined rigidity were set up in 12-well glass bottom plates as reported elsewhere. Briefly, 12 μl of solution (see below) was covered with a cover glass and left for 1 hr at RT to set a gel. After removing the cover glass, the gel was coated with 150 μl of Sulfo-SANPAH Photoreactive Crosslinker (Pierce / ThermoScientific) and activated by UV illumination for 10 min, followed by ECM coating. 5555/4434 cells in DMEM were seeded onto the gels, cultured overnight and treated with drugs for 24 hr. Then, cells were stained with propidium iodide (PI, 1mg/ml) and imaged with a Zeiss 780 inverted microscope. For quantification of cell death, PI positive area (> 500 A.D.U.) was divided by the number of surviving melanoma cells. The composition of gels with defined stiffness and ECM concentration are shown below.

Composition of the gels

Young's modulus	0.2 kPa	3 kPa	12 kPa
10mM HEPES	424.7 μl	403 μl	358 μl
40% PAA	37.5 μl	68.6 μl	94.4 μl
2% Bis-AA	7.5 μl	22.48 μl	40 μl
APS	2.5 μl	2.5 μl	2.5 μl
TEMED	0.25 μl	0.25 μl	0.25 μl

ECM concentration

Type of ECM(s)	Coating	Media
Collagen I	Collagen I (100 mg/ml)	
Fibronectin	Fibronectin (20 mg/ml)	Fibronectin (20 mg/ml)
ECM mixture	Fibronectin (20 mg/ml) Tenascin-C (5mg/ml) TSP-1 (5mg/ml) TSP-2 (5mg/ml)	Fibronectin (20 mg/ml) Tenascin-C (5mg/ml) TSP-1 (5mg/ml) TSP-2 (5mg/ml)

Transfection

For DNA transfection to 5555, 4434, C790, B16F10, A375 and WM266.4 cells, we used lipofectamine 2000 (Invitrogen), and for siRNA transfection to 5555 and 4434, we used lipofectamine RNAiMax (Invitorgen), according to the manufacture's protocol. For DNA transfection to MAFs, we used an electroporation system (Amaxa Nucleofector, Lonza AG).

Immunoblotting

Protein lysates were processed following standard procedures (Calvo et al., 2013), and antibody description and working dilutions used can be found in the following section.

HE, Gomori trichrome stain and immunohistochemistry

Hematoxilin&eosin and Gomori trichrome stain were performed as described elsewhere. For immunohistochemical analysis, the conventional avidin–biotin–peroxidase complex method was used as described previously (Calvo et al., 2013). Images were captured using a Nikon TE2000-S equipped with a Nikon DS-5M camera.

Regents, recombinant proteins, siRNAs and antibodies.

Information about reagents, recombinant proteins, antibodies and siRNAs used in this paper are shown below.

Reagents

Reagent	Company	Cat. No
PLX4720	(Made in house)	
	stratech	S1152
	MedKoo Biosciences	202272
PD184352	(Made in house)	
	LC Labs	P-8499
Imatinib	LC Labs	I-5508
Sunitinib	LC Labs	S-8803
PF573228	Tocris	3239
PF562271	MedKoo Biosciences	202228
FAK inhibitor 14	Tocris	3414
Dasatinib	LC Labs	D-3307
PP2	Calbiochem	529573

Recombinant proteins

Protein	Company	Cat. No
Fibronectin	SIGMA	F1141
CTGF	Peprotech	120-19B
Tenascin-C	Millipore	CC065
Thrombospondin-1	R&D systems	P07996
Thrombospondin-2	R&D systems	P35442
Periostin	R&D systems	Q15063

siRNAs

siRNA	Company	Sequence
QIAGEN CTRL	QIAGEN	Not specified (CODE 1027280)
Mouse ITGB1 smart pool	Dharmacon/Thermo Scientific	GAACGGAUUUGAUGAAUGA CCACAGAAGUUUACAUUAA GCACAGAUCCCAAGUUUCA CAAGAGGGCUGAAGAUUAC
Mouse Ptk2 smart pool	Dharmacon/Thermo Scientific	GAACAUGGCCGACCUGAUU GAAGUUGGGUUGUUUGGAA GAAGAUAAGCCAACAUUGA AGAUGUUGGUUUAAAGCGA

Antibodies

Antibody	Company	Cat. No	Dilution		
			IF	WB	IHC
αSMA	Sigma	A2547	1:200		1: 6000
S100A4 (FSP1)	Abcam	ab27957	1:200		
FAP	Abcam	ab53066	1:200		
Fibronectin	Sigma	F3648	1:200	1:1000	1:1000
Ki-67 (clone TEC-3)	DAKO	M7249			1:125
Integrin β1 (HUTS-21)	BD Pharmingen	556084	1:100		
Integrin β1 (9EG7)	BD Pharmingen	550531	1:100		
Integrin β1	Cell Signaling	#4706		1:1000	
phospho-FAK	Cell Signaling	#8556	1:100		
phospho-FAK	Abcam	ab39967	1:100		
FAK	Cell Signaling	#3285		1:1000	
phospho-ERK	Cell Signaling	#4376		1:1000	1:100
ERK	Cell Signaling	#4695		1:1000	1:100
phospho-MLC	Cell Signaling	#3671		1:1000	
phospho-Src family kinase	Invitrogen	44660G			1:1000
β tubulin	Sigma	T7816		1:200000	
F4/80	Serotec	MCAP497			1:100
S100A9 (2B10)	Abcam	ab105472			1:80
CD3	DAKO	A0452			1:150
CD45R	BD Pharmingen	553086			1:200
Endomucin	Santa cruz	sc-65495			1:100
CTGF	Abcam	ab6992		1:5000	
Tenascin-C	Abcam	ab6393	1:200	1:200	1:4000
Thronbospondin-1	Invitrogen	39-9300	1:100	1:500	
cleaved caspase-3 (Asp175)	R&D systems	MAB835	1:50		

Statistical analysis

Data were subjected to one-way ANOVA analysis, followed by Dunnett's multiple comparison test. When two groups were compared, a two-tailed paired or unpaired Student's *t*-test was applied.

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